

CONSTITUENTS OF UNIONID EXTRAPALLIAL FLUID. I. ELECTROPHORETIC AND IMMUNOLOGICAL STUDIES OF PROTEIN COMPONENTS

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The mechanism of shell formation in mollusks is not well understood (Wilbur and Yonge, 1964). Bivalve shell formation has been shown to occur in a compartment bounded by the shell and the mantle (Bevelander and Nakahara, 1969). The fluid of this compartment, the extrapallial fluid, contains the proteins necessary for the synthesis of an organic matrix able to bind Ca^{++} and $\text{CO}_3^{=}$ ions in a characteristic crystalline configuration (Wilbur and Watabe, 1960). Due to difficulty in working with the extrapallial fluid, the number and nature of the proteins of this fluid is not yet understood. Kobayashi (1964a, 1964b) reported a partial separation of these components from a number of molluscan species using paper and acetate strip electrophoresis. We report a more definitive separation using acrylamide gel electrophoresis. Using extrapallial fluid from several species representing three subfamilies within the family Unionidae, the electrophoretically produced patterns of protein bands and immunological studies reveal a surprising uniformity in the soluble protein composition.

METHODS AND MATERIALS

Mussels

Live mussels were used as the source of extrapallial fluid. Specimens of *Quadrula pustulosa* (Lea), *Quadrula quadrula* (Rafinesque), *Amblema costata* (Rafinesque), *Pleurobema cordatum* (Conrad), *Obliquaria reflexa* (Rafinesque), *Lampsilis silivoides* (Barnes), *L. ventricosa* (Barnes), *Actinonaias carinata* (Barnes), and *Anodonta grandis* (Say), were collected from the Muskingum River at the Beverly, Ohio, bed (Bates, 1970). They were transported to Ypsilanti packed in wet rags and were thereafter maintained in well aerated, circulating tap water at room temperature.

Preparation of samples

Access to the extrapallial fluid was achieved by prying the shell open 2-3 mm, and cutting the adductor muscles. The mantle was pierced near the pallial attachment with an 18 gauge 2-inch needle; then gentle suction was applied to remove the fluid.

Each clam provided enough fluid for a single electrophoresis gel. Normal extrapallial pH for 200 individuals of three species tested was 8.15. Two parts

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fluid were mixed with one part 60% sucrose in a 2.5 ml syringe; then 0.3 ml of this mixture was immediately applied to a gel. Samples were run within five minutes of extraction. Between extractions the syringe was rinsed three times with distilled water to prevent cross contamination.

For injection into rabbits fluid from 3-6 individuals of a single species was spun in a clinical centrifuge to remove the suspended flocculent material, and the supernate was sterilized using a bacterial filter.

Samples of extrapallial fluid to serve as antigen in the agar-gel diffusion studies were pooled from 3-6 individuals and placed directly into the antigen well of the agar plate.

Electrophoresis

A Heathkit 1PW-17 constant voltage power supply was used in conjunction with a Buchler 12-gel capacity water-jacketed electrophoresis chamber. Cold tap water (15° C) was circulated through the water jacket during the operation.

Acrylamide; N, N'-Methylenebisacrylamide (BIS); 2-Amino-2-(hydroxymethyl)-1, 3-propanediol (TRIS); N, N, N', N'-Tetramethylethylenediamine (TEMED); Naphthol Blue Black (Amido Schwartz 10B); Glycine (ammonia free); and Photo-Flo were all obtained from Eastman Kodak and used as supplied. Sucrose (analytical grade) and Ammonium Persulfate (analytical grade) were obtained from the Mallinkrodt Co. and also used as supplied.

Electrophoresis buffer: 28.8 g glycine, and 6.00 g TRIS was diluted to one liter with water. If necessary the solution was adjusted to pH 8.3; Fixative-stain solution: one gram Amido Schwartz per 100 ml 7% (v/v) aqueous acetic acid; Photo-Flo solution: 0.5% (v/v) aqueous Kodak Photo-Flo.

Preparation of acrylamide gels

The procedure of Davis (1964) was followed with the modifications indicated as follows: *Solution A*: 48 ml 1N HCl, 36.6 g TRIS, 0.23 ml TEMED, diluted to 100 ml with water; *Solution B*: 48 ml 1N HCl, 5.98 g TRIS, 0.46 ml TEMED, diluted to 100 ml with water and adjusted with 1N HCl to pH 6.7; *Solution C*: 30.0 g acrylamide, 0.80 g BIS, diluted to 100 ml with water; *Solution D*: 10.0 g acrylamide, 2.5 g BIS, diluted to 100 ml with water; *Solution E*: 40% (w/v) aqueous sucrose; *Solution F*: 0.14% (W/v) aqueous ammonium persulfate; *Solution G*: 0.004% (w/v) aqueous riboflavin.

Separatory gel (small pore)

A mixture of solutions A, C, and F (1, 2, and 4 volumes, respectively) was drawn into a syringe and injected into clean 8.5 cm gel tubes to fill each tube to a height of 4.5 cm. With a 0.5 ml syringe and 26 gauge needle a 2 mm layer of water was placed over the gel. The tubes were allowed to stand 20 minutes as polymerization took place.

Spacer gel (large pore)

The water layer was removed from the surface of the separatory gel, and a mixture was made of solutions B, D, G, and E (1, 2, 1 and 4 volumes respectively), and was injected from a syringe to a height of 6 mm above the separatory gel. A 2 mm layer of water was placed carefully over the spacer gel. The tubes were

placed within one inch of a fluorescent tube for 20 minutes to initiate photopolymerization.

Separation and detection procedures

With the gels in the apparatus and the buffer compartments filled with fresh buffer, the protein-sucrose solutions were layered directly onto the spacer gels. A blank gel was included in each set. All blanks indicated no contamination of upper buffer by extraneous protein. Electrophoresis was performed at 2 mAmp/tube for 15 minutes, then the voltage was turned to the maximum setting, normally producing a current of 6 mAmp/tube, for 50–55 min. The gels were removed from the tubes by squirting water with an 18 gauge needle between the gel and the tube. Each gel was stained overnight in fixative-stain solution and destained electrophoretically in 7% (v/v) aqueous acetic acid at 10 milliAmp per tube for one hour. Gels were then scanned by means of a photometric scanner attached to an Aminco Bowman SPF-125 Spectrophotofluorimeter (Scott and Pietrzak, 1971).

Injection of rabbits for antibody production

Fourteen female virgin New Zealand White Rabbits were injected for antibody production. Injections were every three days as follows: 1 and 2—0.5 ml intravenous, 3—2.0 ml subcutaneous, 4 through 14—0.2 ml subcutaneous plus 0.5 ml Freund Complete Adjuvant (Difco), final—2.0 ml intraperitoneal.

Immunodiffusion

Difco Nobel-Agar was obtained from the Difco Co. and was used as received. Boric acid, sodium tetraborate decahydrate, and sodium chloride were all Baker reagent grade. Merthiolate was obtained from the Lilly Co. and was used as supplied.

Large quantities of whole blood were obtained by cardiac puncture one week after termination of the immunization program. The blood was stored at 4° C for 24 hours to allow clotting. Serum was drawn off with a syringe, made 0.01% in merthiolate to inhibit bacterial growth (Ouchterlony, 1968), and stored frozen at -20° C until use.

Just prior to use, serum was thawed and incubated in a water bath at 56° C for 30 minutes to deactivate the serum complement. Diffusion agar was prepared according to the recommendations of Carpenter (1956). Plastic petri-dishes 5 cm in diameter and 10 mm deep were half filled (6 ml) with agar. Four peripheral wells 7 mm in diameter were punched equidistant from a central well (16 mm center to center). For each cross test 0.1 ml of extrapallial fluid was placed in each peripheral well, and 0.1 ml of antiserum was placed in the center well.

Patterns were allowed to develop in an incubator at 10° C until maximal precipitate arcs formed, (approximately 21 days). In addition, control diffusion-plates using sera from non-inoculated rabbits were run simultaneously with each immuno-system. No immunological response occurred in any control. All tests were run in triplicate, all plates giving identical results within a group.

RESULTS

Table I summarizes the results of electrophoretic separations of soluble extrapallial fluid proteins on acrylamide gels. The A band because of its dependability

TABLE I
Electrophoretic results

Species*	Number of specimen	Type of Data†	Bands observed											
			A	B	C	D	E	F	G	H	I	$\alpha 1$	$\alpha 2$	$\alpha 3$
Qq	25	M	1.00	0.70	0.59	0.50	0.40	0.35	0.30	—	0.14	1.40	1.63	2.15
		SD	—	± 0.013	± 0.012	± 0.012	—	± 0.009	—	—	± 0.013	± 0.000	± 0.050	± 0.050
Qp	20	P	100	88	96	100	36	4	96	0	72	40	24	17
		M	1.00	0.70	0.059	0.49	0.38	0.35	0.29	—	0.13	1.40	—	—
Ac	30	SD	—	± 0.012	± 0.010	± 0.017	± 0.011	± 0.012	± 0.010	—	± 0.019	—	—	—
		P	100	95	15	85	24	56	60	0	60	4	0	0
Pc	4	M	1.00	0.66	0.59	0.50	0.39	0.35	0.30	0.26	0.15	—	—	—
		SD	—	± 0.012	± 0.013	± 0.011	± 0.010	± 0.010	± 0.011	± 0.013	± 0.016	—	—	—
Or	3	P	100	87	42	94	42	13	74	49	87	0	0	0
		M	1.00	0.80	0.60	0.50	0.39	0.34	0.30	0.25	0.16	—	—	—
Ls	4	P	100	75	75	100	100	50	75	50	100	0	0	0
		M	1.00	0.72	—	0.49	0.40	0.35	0.32	0.26	0.14	—	—	—
Ls	4	P	100	67	0	100	33	67	33	100	100	0	0	0
		M	1.00	0.80	—	—	—	0.38	—	0.30	—	—	—	—
Ls	4	P	100	25	0	0	0	50	0	75	0	0	0	0
		M	1.00	0.80	—	—	—	—	—	—	—	—	—	—

* Q₄ = *Quadrula quadrula*, Q_p = *Quadrula pustulosa*, Ac = *Ambelma costata*, Pc = *Pleurobema cordatum*, Or = *Obliquaria reflexa*, Ls = *Lampsilis siliquidea*.

† M = mobility referenced to A = 1.00, SD = Standard deviation of mobility, P = Percentage of samples displaying the band.

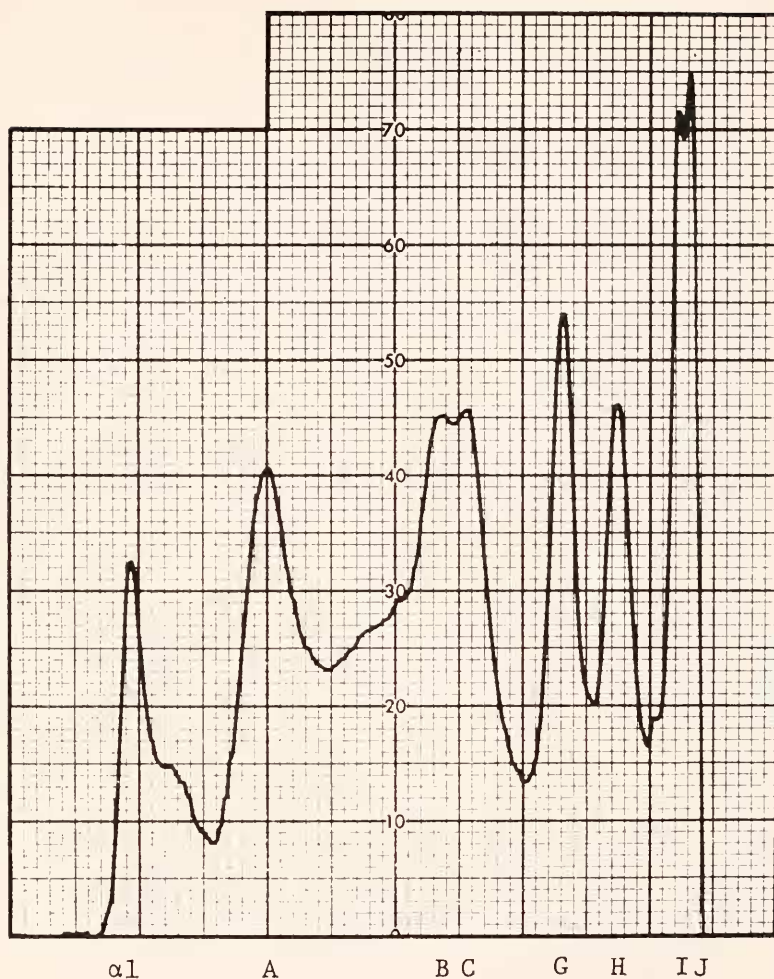


FIGURE 1. A typical densitometer scan of an acrylamide gel electrophoretic separation of extrapallial fluid.

of occurrence, was assigned a relative mobility of 1.00, and all other bands are reported as fractions or multiples of this value.

All gels were scanned by a densitometer, a typical result being shown in Figure 1. This technique proved superior to visual inspection for the detection of individual bands and facilitated accurate calculations of mobilities. Due to background coloration and overlapping of bands, no attempt was made to integrate the areas under the peaks as a measure of total protein in each band. It is obvious from inspection of the scans, however, that bands A, E, F, and G represent proteins present in relatively high concentration. Bands B, C, D, and I represent compounds present in moderate concentration, while the α bands are too diffuse to allow a reasonable estimation of concentration to be made. When present the H band could be either

intense or weak. The J band was too slow moving to permit a measurement of its mobility. In fact, considering its position at the end of the gel it could well be an artifact.

The frequency of occurrence of each band was considered. The A band was particularly significant being present in good concentration in every organism studied. Bands B, D, G, and I were generally present, while bands C, F, and H were less dependable and showed considerable species variation. For example, band C was virtually always present in *Quadrula quadrula* but seldom present in *Quadrula pustulosa*. There was only one individual occurrence of an α band outside of *Quadrula quadrula*, and even in that species they appeared in less than half of the samples assayed.

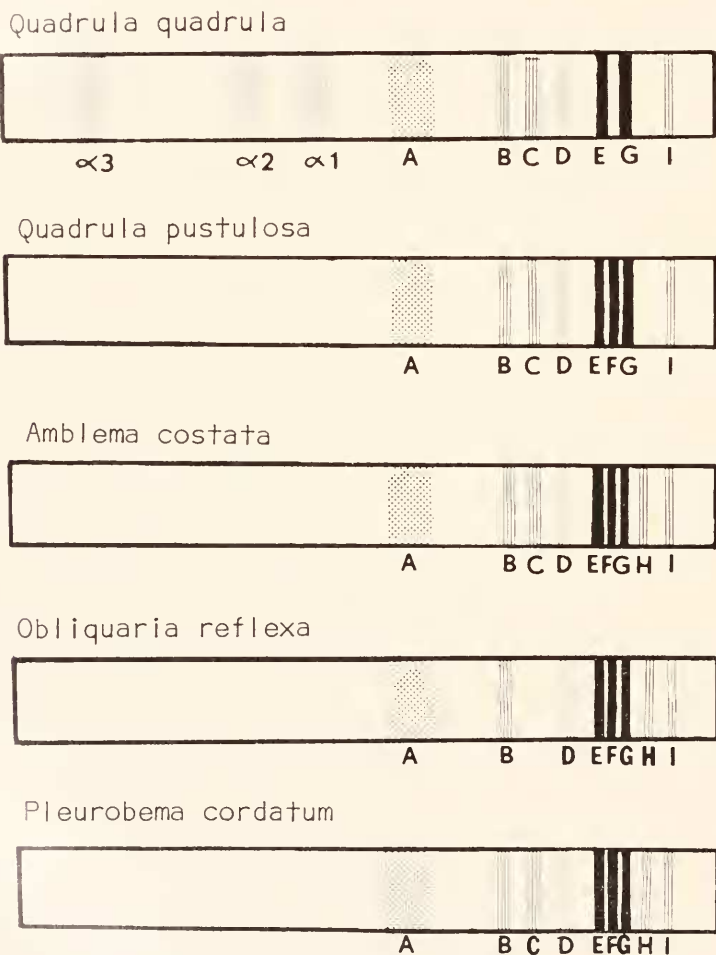


FIGURE 2. Patterns for five species showing the relative positions of frequently occurring bands.

The size and age of each specimen was recorded and correlated with the assay of its fluid. Examination of this data failed to reveal any relationship between age and the frequency of appearance of any particular band.

It is noteworthy that band B showed a much greater variation in mobility from one species to another than any of the other bands. Where large samplings were taken, it showed a very reproducible value for the particular species. Typical band patterns for five species are presented for comparison in Figure 2.

On the basis of the interpretation of the acrylamide gel data, immunological studies to ascertain if the similar patterns in fact represented identical protein species were undertaken.

An "F" reaction is defined as a cross reaction between homologous and heterogenous systems with deviation and complete fusion of the immuno-precipitate arc as in an Ouchterlony "Identity" reaction (Ouchterlony, 1968). A "C" reaction is defined as a cross reaction between homologous and heterogenous systems in which the immuno-precipitate arc was of insufficient density or extension to absolutely establish an "F" type reaction. The "C" reaction was attributed to lower antigen or antibody concentration in the complex systems tested. Table II indicates the results of test crosses between homologous and heterogenous immuno-systems. Cross reactions occurred without exception in all test crosses of heterogenous antigen and homologous systems among species from all three subfamilies. The cross reactions were often characterized by Ouchterlony "Identity" reactions—deviation and complete fusion of precipitate arcs. The generally accepted interpretation of this reaction is that the antigens have a factor in common and are, due to this determinant, precipitated by the corresponding antibody component to this

TABLE II

Results of test crosses between homologous and heterogenous extrapallial fluid immuno-systems.

Symbols are: A-Qq, A-Qp, A-Ac, A-Xc, A-Ls, A-Ag are the antisera and Qq, Qp, Ac, Xc,

Ls, Ag are the antigens corresponding to Quadrula quadrula, Quadrula pustulosa,

Amblema costata, Actinonaias carinata, Lampsilis siliquoidea, and Anodonta

grandis. Lv is the antigen corresponding to Lampsilis ventricosa. F and C

refer to F type and C type immuno-reactions, while the number in

brackets indicates the number of bands of the particular reaction.

N.R. indicates that the combination was not tried.

Cross test antigen	Homologous antibody—antigen					
	1	2	3	4	5	6
	A-Qq + Qq	A-Qp + Qp	A-Ac + Ac	A-Xc + Xc	A-Ls + Ls	A-Ag + Ag
Qq	F (1)C (2)	F (4)	F (1)C (1)	F (2)	F (1)	C (2)
Qp	F (1)C (1)	F (4)	F (1)C (1)	F (1)C (1)	C (2)	C (2)
Ac	C (2)	F (1)C (1)	F (1)C (1)	C (1)	C (2)	C (2)
Xc	N.R.	N.R.	N.R.	F (2)	N.R.	N.R.
Ls	C (3)	C (2)	F (1)	F (1)	F (1)C (1)	N.R.
Lv	N.R.	N.R.	N.R.	N.R.	F (1)C (1)	C (2)

common determinant. These "F" type reactions were evident in test crosses between heterogenous antigen in 12 of 21 crosses.

DISCUSSION

The shell matrix has been established to be protein in nature. Gregoire, Duchateau, and Florkin (1955) reported the presence of a polypeptide and two proteins, nacrine and nacroscerotine, in this material. X-ray diffraction patterns indicated an α or β keratin structure (Wilbur and Watabe, 1963). Within the CaCO_3 crystal both water soluble and insoluble components were found (Gregoire, 1955). The non-dialyzable soluble fraction was found to precipitate with trichloroacetic acid, inferring a protein substance. This was found in each of thirty-two species of gastropod tested and accounted for nearly half of the total matrix protein in some of these species (Florkin and Stotz, 1968).

A recent elegant study (Bevelander and Nakahara, 1969) proposed a mechanism for shell formation based on shell electron micrographs. A compartment was described as forming near the surface of the shell, attaching to the shell and trapping a modified extrapallial fluid. The compartment fills with fibers and granules, this being followed by the formation of carbonate crystals. The envelope described is presumably protein in nature, probably what has been described elsewhere as the shell matrix. The protein precursors thus incorporated and the enzymes necessary for these transformations must have originated in the extrapallial fluid.

Previous studies with marine species (Gregoire, 1957, 1960) involving electron micrographs of shell matrix revealed that patterns and sizes of perforations are differentially characteristic of Gastropoda, Pelecypoda, and Cephalopoda. Hare and Abelson (1965) demonstrated that the amino acid compositions of matrix proteins are strikingly similar among species within a single family of Pelecypoda, but that differences are significant between families.

Gel electrophoresis provided resolution of the protein components of the extrapallial fluid superior to that previously reported with paper or cellulose acetate strips (Kobayashi, 1964). Evidence presented in this report supports the concept that within a single species there are some proteins which appear in all or virtually all samples of extrapallial fluid. Other proteins occur in a more variable manner from one individual to the next. This is not inconsistent with the concept of a functioning system maintaining a constant supply of some components and generating others as necessary. Conceivably some enzymes and a supply of the basic unit of shell matrix protein might be present as stable components while other enzymes or matrix intermediates vary according to the current rate or stage in shell formation by the individual. The similarity of the patterns from one species to another supports the concept that the mechanism of shell formation may well be very similar or identical among those species.

In terms of the immunological evidence, it is not possible to determine the nature of the proteins or conjugated proteins found in the fluid. Further, it is not intended to reveal the absolute number of components involved since the acrylamide data provides superior resolution in this regard. It is possible to state that definite close antigenic relationships do exist among some components of extrapallial fluid of both closely and distantly related species of the family Unionidae. These similarities manifest themselves as extensive cross reactions between heterogenous immunosystems and are characterized by frequent "F" type interactions.

This study was supported in part by United States Bureau of Commercial Fisheries Contract No. 14-17-0004-433, Sub Project No. 4-28-R, State of Ohio.

SUMMARY

Components of extrapallial fluid of several species within the three sub-families of the family Unionidae have been comparatively analyzed. It has been found that the proteins involved in shell formation for these species are strikingly similar, electrophoretically and immunologically. These data infer that the same protein components may well be involved in the formation of shell within species of the family Unionidae.

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